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Antibacterial activity of *Eleutherine bulbosa* against multidrug-resistant bacteria

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Abstract

Background: Recent interest in plant-based medicine is justified, as synthetic antioxidants and antibiotics suffer from several drawbacks. Plant extracts have been shown to possess health promoting properties, and they exert no selective physiological pressure on the pathogens that can result in development of drug resistance. The aim of this study was to investigate the antibacterial activity of *Eleutherine bulbosa* plant extracts against a series of multidrug-resistant (MDR) bacteria by calculating zones of inhibition, minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and time–kill kinetics.

Methods: Dried bulb powder of *E. bulbosa* was extracted with five solvents (ethyl acetate, chloroform, butanol, ethanol and water) to select the best extractant for subsequent isolation of bioactive compounds to use against a battery of MDR bacteria.

Results: Butanol was found to be the best extractant with an MIC ranging from 46 μg/mL to 187 μg/mL. Butanol and aqueous extracts showed higher zones of inhibition in comparison with standard antibiotics gentamicin and ciprofloxacin. Thin layer chromatography (TLC)-bioautography was carried out with the butanol extract against *Staphylococcus aureus* and *Shigella boydii*. A synergistic effect was established against *S. boydii* by combining the chloroform extract with gentamicin.

Conclusion: The bulb extracts of *E. bulbosa* exhibited significant antibacterial activity. Further study is necessary to characterize the antibacterial compounds of this plant for use in clinical applications.

Keywords: Multidrug-resistant bacteria; time–kill kinetics; TLC-bioautography

1. Introduction

Worldwide, infectious diseases are killing almost 50,000 people every day.[1] Commercial antimicrobial drugs are commonly used for their treatment, which can lead to several side effects. A number of plants are also used in the treatment of a wide variety of infectious diseases. The extensive use of commercial antibiotics, both for human consumption and animal production, has promoted the development of resistance in a variety of pathogenic bacteria.[2] The emergence of bacterial strains that exhibit resistance to a collection of antibiotics, i.e., strains that are multidrug resistant (MDR), is becoming a major cause of treatment failure of infections worldwide. Furthermore, increasing resistance may lead to reoccurrence of infectious diseases (e.g., tuberculosis and pneuminia) that were once thought to be under control in developed countries. Incidence of MDR in Gram-positive (*Staphylococcus* and *Bacillus*), Gram-negative (*Escherichia, Pseudomonas, Shigella*, and *Salmonella*), and other bacteria has been reported concurrently from all over the world. This is due to the indiscriminate use of synthetic antibiotics. It has renewed the interest of researchers and academics in the development of antibiotics from medicinal and aromatic plants, as the plant products are safe, do not produce any side effects, do not exert any physiological pressure on the pathogens for the development of drug resistance, are easily

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degradable, nonaccumulative in the environment and do not cause environmental pollution.[3]

Eleutherine bulbosa (Miller) Urban is an herbaceous plant of the Iridaceae family. Bulbs are ovoid, 0.5–3.5 cm in diameter with several layers of brittle, red brown tunics. The leaves (1–4 in number) are narrowly lanceolate [dimensions of 19–36 cm × 0.5 cm × 1.4 (2–3) cm], prominently veined (5–7 veins) with a flower stem of 22–30 cm in length and conduplicate floral bracts, the outer being herbaceous and the inner largely membranous. Flowers are 2.5 cm in diameter with obovate perianth lobes. Flowers open in the evening and last only a few hours. This plant is native to South America and is present in tropical countries. The bulbs are used in folk medicine as an abortifacient and emmenagogue,[4] to treat heart failure,[5] as a purgative and an anticancer drug.[6,7] Recently, Ha et al[8] found that the methanol extract of the rhizome of *E. bulbosa* potently inhibited the lipopolysaccharide (LPS)-stimulated productions of interleukin-12 p40 (IL-12 p40) and IL-6 cytokines in bone marrow-derived dendritic cells.

In the course of preliminary screening of medicinal plants of the Mayurbhanj District (India) for antimicrobial activity, we found that the methanol extract of the bulb had strong activity against both fungi and bacteria.[9] Therefore, a detailed study was conducted to investigate the antibacterial activity of *E. bulbosa* against a series of MDR bacteria by calculating zone of inhibition, minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and time–kill kinetics. Moreover, the antibacterial properties of the extract were evaluated by combining it with commercial antibiotic to determine the synergistic/antagonistic effects. Further, the bulb extracts were subjected to thin layer chromatography (TLC) and TLC-bioautography studies.

2. Materials and methods

2.1. Collection and identification of plant material

The bulbs of *E. bulbosa* were collected from a forest guest house in Baripada, Mayurbhanj, Odisha, India. Identification and voucher specimen deposition of this plant was performed at the Department of Botany, North Orissa University, Baripada, Odisha, India.

2.2. Processing of plant material and preparation of crude extracts

The bulbs were shed dried, followed by drying in a hot dry oven for 1 hour at 60°C. The sample was powdered using a mechanical grinder. Solvent extraction using the maceration method was carried out by soaking 500 g of dried plant material in cleaned airtight bottles of chloroform at a temperature of 28°C for a period of 2 days. After proper incubation, the solvent was filtered using filter paper. The filtration process was repeated twice and the extracts obtained were pooled for evaporation to obtain residue. Further studies were carried out by treating the residue with different solvents including ethyl acetate, butanol, and ethanol as outlined in Figure 1. Plant extracts obtained by maceration were concentrated and all the residues obtained were stored in airtight vials at 4°C. For aqueous extraction, 100 g of powdered bulb sample were placed separately in a conical flask containing 400 mL of distilled water, followed by steam distillation for 30 minutes to reduce the aqueous extract to one fifth of the starting volume.

2.3. Bacterial strains

Bacterial cultures were obtained from government institutes of India such as Institute of Microbial Technology, Chandigarh (MTCC-The Microbial Type Culture Collection and Gene Bank), Regional Medical Research Centre, Bhubaneswar (RMRC) and National Institute of Cholera and Enteric Diseases, Kolkota (NICED). Pathogenic organisms under study included 11 Gram-negative bacteria (*Escherchia coli* MTCC 1098, *Escherichia coli* O157:H7 RMRC, *Pseudomonas aeruginosa* MTCC 1034, *P. fluorescens* MTCC 1748, *Salmonella typhimurium* MTCC 3216, *Shigella boydii* RMRC, *S. sonnei* NICED, *S. flexneri* NICED, *S. dysentriae* NICED, *Vibrio alginolyticus* MTCC 4439, *V. cholerae* MTCC 3904) and five Gram-positive bacteria (*Bacillus brevis* MTCC 3904, *B. licheniformis* MTCC 7425, *B. subtilis* MTCC 7164, *Staphylococcus aureus* MTCC 1144, *S. epidermidis* MTCC 3615). All these strains were grown at 37°C and maintained at 4°C on nutrient agar slants (HiMedia Laboratories Ltd., Mumbai, India).

2.4. Antibiotic sensitivity test

An antibiogram was generated by a disk diffusion method[10] in Mueller-Hinton (MH) agar with commonly used antibiotics (Figure 2). All these antibiotics were procured from HiMedia Laboratories Ltd. To determine the antibiotic sensitivity profiles of reference bacteria (Figure 3), the antibiotics were used at the following specific concentrations: amoxicillin (10 μg); ampicillin (10 μg); bacitracin (10 units); cefoxitin (10 μg); ceftriaxone (10 μg); cephotaxime (30 μg); chloroamphinecol (10 μg); ciprofloxacin (10 μg); erythromycin (15 μg); gatifloxacin (30 μg); gentamicin (10 μg); levofloxacin (5 μg); naladixic acid (30 μg); ofloxacin (5 μg); polymyxin-B (300 units); streptomycin (10 μg); tetracycline (10 μg) and vancomycin (30 μg).

2.5. Assay for antimicrobial activity by agar cup method

The agar cup method was used to study the antibacterial activity of the extracts. Overnight MH broth culture of the test organisms were firmly seeded over the MH agar plates. Wells approximately 6 mm in diameter and 2.5 mm in depth were made on the surface of the solid medium using a sterile borer. Each well was filled with 40 μL of test sample (30 mg/mL for each extract). Respective solvents without plant extracts served as controls while standard antibiotics—40 μL of gentamicin (10 μg/mL) and ciprofloxacin (10 μg/mL)—were used as reference controls. The plates were incubated at 37°C for 24 hours. After 24 hours, the plates were removed and

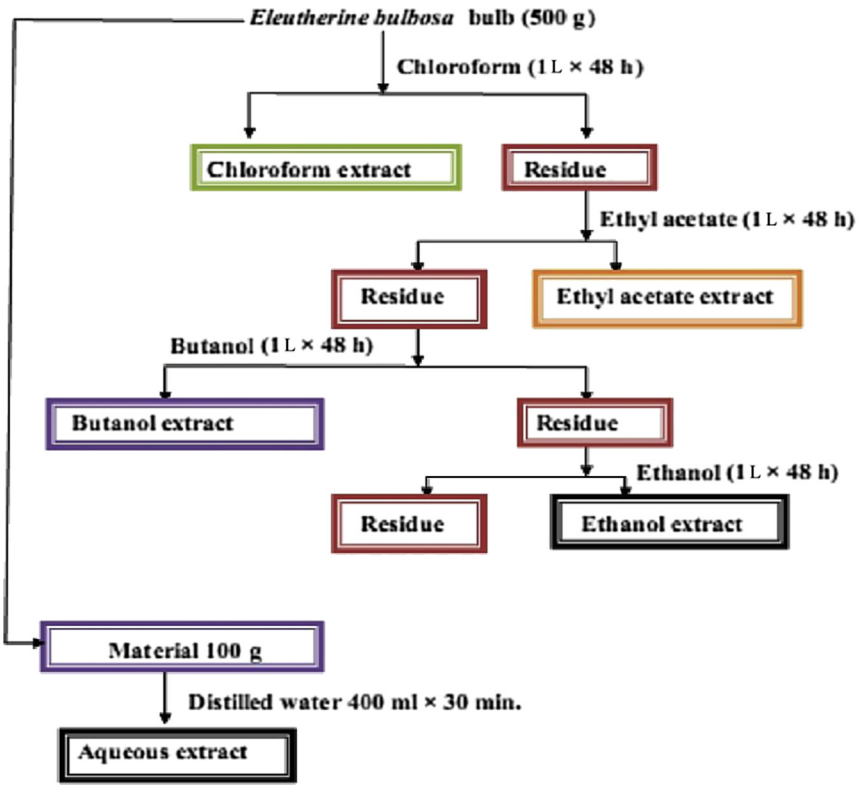

Figure 1. Schematic representation of the maceration and refluxing extraction from *Eleutherine bulbosa*.

zones of inhibition measured with a HiMedia antibiotic scale and the results were tabulated. Extracts with zones of inhibition ≥8 mm in diameter were regarded as positive. Each experiment was carried out in triplicate. The mean ± standard deviation (SD) of the inhibition zone was used for calculating the antimicrobial activity of the extracts.

2.6. Evaluation of MIC

The broth microdilution technique was adopted using 96-well microtiter plates (Figure 4) with tetrazolium salt (2,3,5-triphenyltetrazolium chloride, TTC) as an indicator to determine the MIC. Crude extract was added to the second

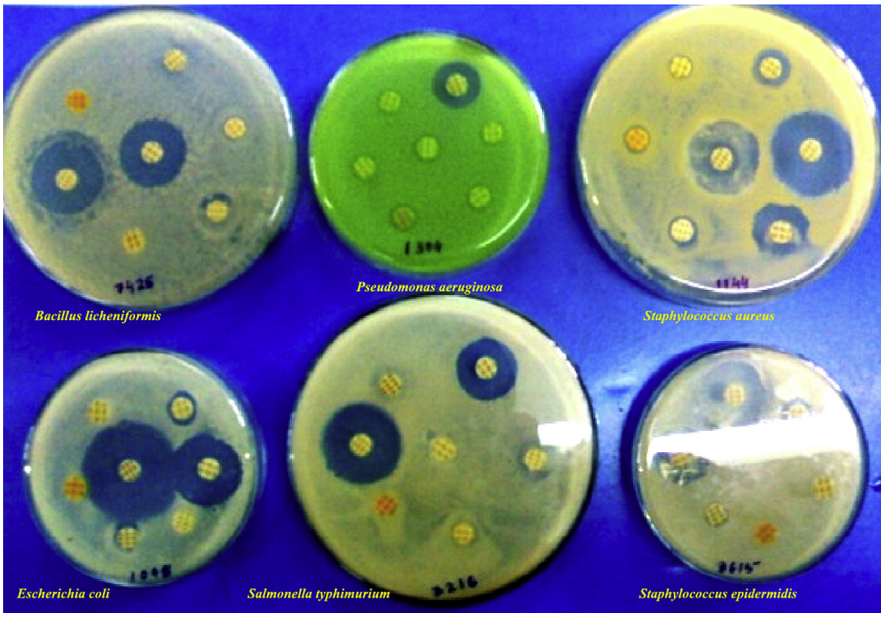

Figure 2. Screening of antibiotic sensitivity among bacteria by a disk diffusion method.

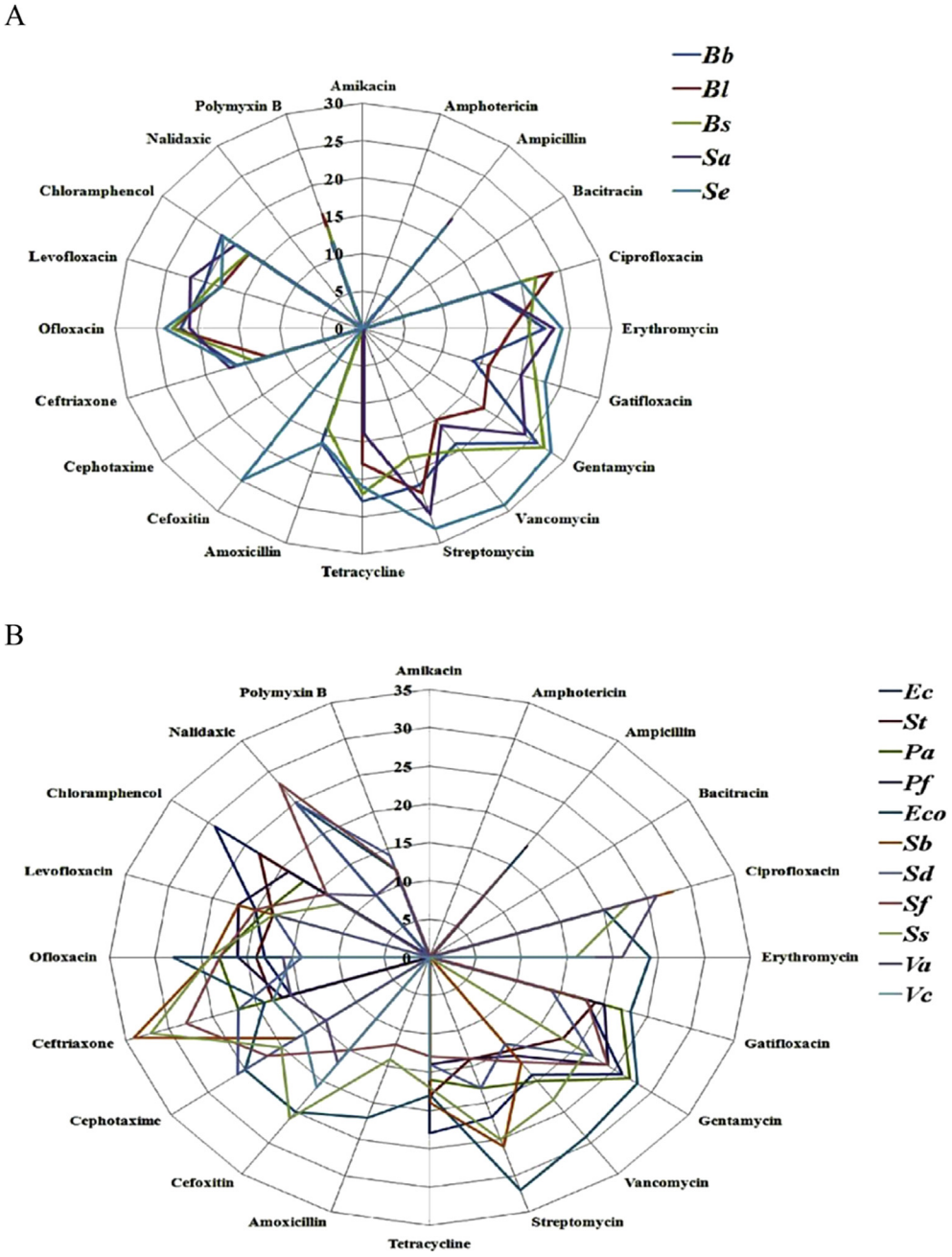

Figure 3. Antibiogram for (A) Gram-positive and (B) Gram-negative bacteria in our bacterial cohort. Bb = *Bacillus brevis*; Bl = *Bacillus licheniformis*; Bs = *Bacillus subtilis*; Ec = *Escherichia coli*; Eco = *Escherichia coli* O157:H7; Pa = *Pseudomonas aeruginosa*; Pf = *Pseudomonas fluorescens*; Sa = *Staphylococcus aureus*; Sb = *Shigella boydii*; Sd = *Shigella dysentriae*; Se = *Staphylococcus epidermis*; Sf = *Shigella flexneri*; Ss = *Shigella sonnei*; St = *Salmonella typhimurium*; Va = *Vibrio alginolyticus*; Vc = *Vibrio cholerae*.

column (A2-H2) of the 96-well plate and serial dilution performed as described previously.[11] The microtiter plates were sealed with parafilm and incubated for 24 hours at 37°C with agitation (130 rpm) and observed for growth of the bacteria. A visible color change to pink (Figure 4) indicated growth of bacteria. The MIC value of the extract was taken as the lowest concentration that showed no growth for individual test bacteria.

2.7. Calculation of MBC and total activity

A sample of 10 µL of the broth from each well of the 96-well microtiter plate exhibiting MIC and from control wells was taken aseptically and plated on MH agar plates as a spot inoculum under the laminar flow hood. These plates were then sealed and incubated at 37°C for 24 hours and observed for growth of the bacteria. Absence of bacterial growth

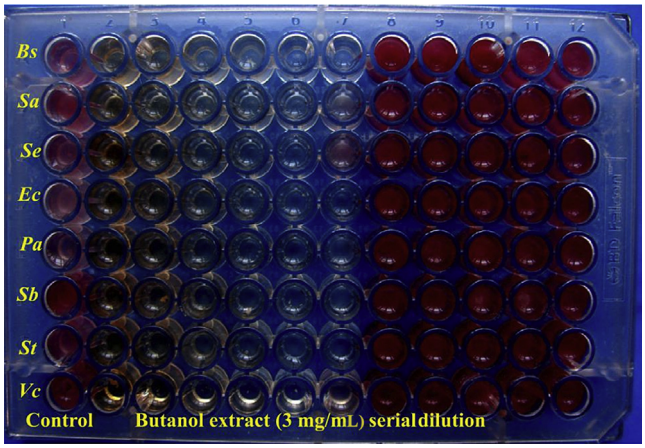

Figure 4. Determination of the minimum inhibitory concentration of *Eleutherine bulbosa* bulb extracts against a number of bacteria in a 96 well microtiter plate. Development of pink color corresponds to growth of bacteria in the presence of 2,3,5-triphenyltetrazolium chloride. Bs = *Bacillus subtilis*; Ec = *Escherichia coli*; Pa = *Pseudomonas aeruginosa*; Sa = *Staphylococcus aureus*; Sb = *Shigella boydii*; Se = *Staphylococcus epidermis*; St = *Salmonella typhimurium*; Vc = *Vibrio cholerae*.

indicated the MBC for the respective bacteria. Total activity is a measure of the amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. In mathematical terms it can be expressed as: Total activity = Amount extracted from 1 g (mg) or amount present in fraction (mg)/MIC (mg/mL). The total activity is expressed in mg/g and indicates the degree to which the active extracts, fractions or compounds present in 1 g can be diluted and still inhibit the growth of the test organisms.

2.8. In vitro toxicological study using *Pseudomonas fluorescens* assay

Microbial toxicity was screened using *P. fluorescens* as a model organism, as described by Panda.[12] An aliquot of 100 mL of nutrient broth was inoculated with the bacterial culture and incubated overnight at room temperature in an orbital shaker. Subsequently, 200 mL of fresh nutrient broth was inoculated with 10 µL of the overnight culture. This suspension was used as test inoculum. Dilutions of different extracts ranging from 1.875 µg/mL to 750 µg/mL were prepared in sterile distilled water. Samples (5 mL) of each dilution were mixed with 5 mL of test inoculum in a 15 mL culture tube; then 2 mL of the sample was taken from each tube and the optical density (OD) was recorded at zero hours at 660 nm. The tubes were incubated, along with controls, at 37°C for 18 hours. Then 2 mL from each dilution flask was removed and the OD was recorded at 660 nm. The percentage of inhibition was calculated using the following formula: % of inhibition = 100*S/C, where S (Sample) = O.D. of *P. fluorescens* at 18 hours with extracts - O.D. of *P. fluorescens* at 0 hours with extracts and C (Control) = O.D. of *P. fluorescens* at 18 hours without extracts - O.D. of *P. fluorescens* at 0 hours without extracts. Effective concentration for 50% inhibition (EC_{50}) was determined by plotting a graph of the percentage of inhibition versus concentration of sample.

2.9. Time–kill kinetics

The chloroform extract was further subjected to time–kill kinetics with *S. boydii*. A 20-µL sample of overnight broth culture of *S. boydii* was added to 180 µL of MH broth containing chloroform extract. The microtiter plate was incubated at 37°C.

Table 1
Screening of the antibacterial activity of the *Eleutherine bulbosa* bulb.

St. no.	Name of the strain	Zone of inhibition[a] (mm)						
		Ethyl acetate	Chloroform	Butanol	Ethanol	Aqueous	G	C
Gram-negative								
1	*Escherichia coli*	15.6 ± 1.5	11.3 ± 1.58	19.0 ± 2.6	12.3 ± 0.58	13.3 ± 1.15	24.0 ± 1.73	19.6 ± 2.08
2	*Escherichia coli* O157:H7	13.3 ± 1.15	—	26.0 ± 1.4	—	26.7 ± 2.08	21.5 ± 0.57	23.0 ± 2.0
3	*Pseudomonas aeruginosa*	17.0 ± 1.4	—	21.0 ± 1.4	13.3 ± 2.08	18.3 ± 1.15	33.6 ± 1.52	17.1 ± 1.15
4	*Pseudomonas fluorescens*	14.0 ± 1.0	—	22.0 ± 2.6	12.3 ± 0.58	16.7 ± 2.08	14.7 ± 1.15	19.0 ± 2.64
5	*Shigella boydii*	14.7 ± 0.58	—	24.0 ± 2.6	11.3 ± 1.58	13.0 ± 1.0	—	18.0 ± 1.0
6	*Shigella dysentriae*	13.0 ± 1.73	—	24.3 ± 2.5	11.7 ± 1.53	14.7 ± 0.58	20.0 ± 2.0	16.3 ± 1.05
7	*Shigella flexneri*	17.3 ± 1.15	—	15.0 ± 3.2	—	15.3 ± 1.15	19.3 ± 2.3	23.6 ± 1.10
8	*Salmonella typhimurium*	13.3 ± 2.08	11.3 ± 1.58	20.0 ± 1.4	13.3 ± 2.08	20.0 ± 1.4	23.0 ± 1.0	24.6 ± 1.53
9	*Shigella sonnei*	18.0 ± 1.0	13.3 ± 2.08	15.0 ± 2.6	12.3 ± 0.58	18.0 ± 2.6	14.7 ± 1.53	26.6 ± 1.10
10	*Vibrio alginolyticus*	14.7 ± 0.58	11.7 ± 1.53	23.0 ± 1.4	13.0 ± 1.73	22.0 ± 2.6	—	19.3 ± 2.51
11	*Vibrio cholerae*	12.7 ± 0.58	16.0 ± 0.0	18.0 ± 1.4	12.7 ± 0.58	21.0 ± 1.4	—	22.3 ± 1.15
Gram-positive								
12	*Bacillus brevis*	20.0 ± 1.4	13.3 ± 2.08	23.0 ± 1.4	15.6 ± 1.5	—	18.3 ± 1.53	26.6 ± 0.57
13	*Bacillus subtilis*	14.0 ± 2.0	12.7 ± 2.52	22.0 ± 2.6	15.0 ± 2.0	18.0 ± 1.4	21.0 ± 1.00	16.6 ± 1.52
14	*Bacillus licheniformis*	18.3 ± 0.5	13.0 ± 1.73	18.0 ± 1.4	18.3 ± 0.5	18.0 ± 2.6	22.3 ± 1.52	15.6 ± 0.57
15	*Staphylococcus aureus*	14.5 ± 2.6	13.7 ± 1.15	24.0 ± 0.0	14.5 ± 2.6	20.0 ± 2.6	—	29.0 ± 2.0
16	*Staphylococcus epidermidis*	15.0 ± 2.0	13.7 ± 2.08	29.0 ± 2.6	14.0 ± 2.0	24.3 ± 2.5	—	26.6 ± 1.52

— = no zone of inhibition; C = ciprofloxacin; G = gentamicin; St. no. = strain number.
[a] Values represent mean zone of inhibition ± standard deviation (SD; mm). Zone of inhibition includes 6 mm borer. The extract concentration is 30 mg/mL.

The number of viable cells was determined after 0 hours, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 16 hours, 24 hours, and 48 hours of incubation. A control culture without crude extract was incubated and assayed under the same conditions. Synergy was more likely to be expressed when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was the same for all components of the mixture. The fractional inhibitory concentration index (ΣFIC) was calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the ΣFIC is ≥0.5, and antagonistic when the ΣFIC is ≤2.[13]

2.10. TLC-bioautography

TLC-bioautography assays were performed by an agar overlay bioautography technique.[14] Plant extract samples (5 μL) were applied 2.5 cm from the base of the silica plate. After drying, the plates were developed using solvent chloroform/methanol (9:1). Developed TLC plates were carefully dried for complete removal of solvents. Aliquots of 50 mL of molten MH agar was prepared by adding 500 μL of bacterial inoculum (5×10^5 colony forming units; CFU). The inoculum-containing agar was overlaid on dried TLC plate under aseptic conditions in a laminar flow hood. The plates were incubated at 37°C and examined for the zone of inhibition.

3. Results

The antibacterial activity was evaluated by using the agar cup method against 16 human pathogenic bacteria (Table 1). In the agar cup method, the test bacteria with a confirmed zone of inhibition were found with ethyl acetate, ethanol, butanol and aqueous extracts. The yields were 26.4%, 16.3%, 11.3%, 6.4% and 5.4% in ethanol, aqueous, butanol, chloroform and ethyl acetate extracts, respectively. The MIC results among Gram-positive and Gram-negative bacteria (Table 2) showed that ethyl acetate, butanol, ethanol, and aqueous extracts were able to prevent the growth of most of the test strains with selective activities. The concentration for inhibition of growth of the test bacteria ranged from 46 μg/mL (w/v) to 3000 μg/mL (w/v) with the lowest MIC value against *B. brevis*, *B. licheniformis*, *S. boydii*, *S. sonnei* (46 μg/mL) followed by *B. subtilis*, *S. epidermidis*, *S. aureus*, *P. aeruginosa*, *V. alginolyticus*, *S. typhimurium*, *E. coli*, *E. coli* 0157:H7 (94 μg/mL) while *P. fluorescens*, *S. dysentriae*, *S. flexneri* and *V. cholerae*, were inhibited at 187 μg/mL. The results of the MBC test showed that with a concentration of 3000 μg/mL (w/v), 50% of the organisms from both Gram-negative and Gram-positive bacteria were killed. Toxicological screening was carried out using *Pseudomonas fluorescens* and effective concentrations were determined (Table 3). The EC_{50} values were 69.11 μg/

Table 3
Evaluation of the toxicological effects of *Eleutherine bulbosa* bulb extracts using *Pseudomonas fluorescens*.

Extract concentration (μg/mL)	% of inhibition and EC_{50} of each extract				
	Ethyl acetate	Chloroform	Butanol	Ethanol	Aqueous
750	89.42	74.25	94.96	80.92	93.6
600	76.52	69.24	89.05	77.56	82.5
450	66.23	64.12	66.45	65.04	74.1
300	59.86	55.46	59.42	56.02	62.05
150	56.04	47.52	58.52	54.08	59.05
75	54.26	40.51	54.51	53.02	54.48
15	46.04	41.2	51.2	43.22	50.07
7.5	38.81	31.03	47.03	40.55	42.5
3.75	29.05	28.2	42.2	32.05	43.4
1.875	23.06	18.04	38.04	28.4	32.5
0	0	0	0	0	0
EC_{50}	69.11	270.46	14.64	70.72	14.79

EC_{50} = effective concentration for 50% inhibition.

Table 2
Results of MIC and MBC assays of *Eleutherine bulbosa* bulb extracts.

St. no.	Ethyl acetate			Chloroform			Butanol			Ethanol			Aqueous		
	MIC[a]	MBC[a]	TA[a]	MIC[a]	MBC[a]	TA[a]	MIC[a]	MBC[a]	TA[a]	MIC[a]	MBC[a]	TA[a]	MIC[a]	MBC[a]	TA[a]
1	187	1500	288.8	1500	>6000	42.7	94	750	1202	187	750	1411	375	1500	434.7
2	187	1500	288.8	750	>6000	85.3	94	750	1202	187	750	1411	375	1500	434.7
3	094	750	574.5	3000	>6000	21.3	94	750	1202	1500	>6000	176	94	750	173.4
4	187	1500	288.8	3000	>6000	21.3	187	750	604.3	1500	>6000	176	375	1500	434.7
5	94	750	574.5	3000	>6000	21.3	46	750	2456	1500	3000	176	46	750	5343.4
6	187	1500	288.8	3000	>6000	21.3	187	750	604.3	1500	3000	176	375	1500	434.7
7	187	1500	288.8	3000	>6000	21.3	187	750	604.3	750	>6000	19.0	375	1500	434.7
8	94	750	574.5	750	>6000	246	94	750	1202	750	>6000	352	94	750	173.4
9	94	750	574.5	750	>6000	85.3	46	750	2456	750	3000	352	46	750	5343.4
10	187	750	288.8	750	>6000	85.3	94	750	1202	750	3000	352	187	1500	871.6
11	187	1500	288.8	375	>6000	246	187	750	604.3	1500	>6000	176	187	1500	871.6
12	46	375	1174	375	>6000	170.7	94	750	1202	1500	3000	176	750	1500	217.3
13	187	750	288.8	375	>6000	170.7	94	750	1202	1500	3000	176	94	750	173.4
14	187	750	288.8	3000	>6000	21.3	46	750	2456	1500	3000	176	94	750	1734
15	187	750	288.8	375	>6000	170.7	187	750	604.3	1500	3000	176	94	750	1734
16	187	750	288.8	375	>6000	170.7	46	750	2456	1500	3000	176	94	750	1734

MBC = minimum bactericidal concentration; MIC = minimum inhibitory concentration; St. no. = strain number; TA = Total activity.
[a] Values are expressed in μg/mL.

Table 4
The R_f values of different components of *Eleutherine bulbosa* bulb extracts.

Extract	Mobile phase	Number of spots (by UV)	R_f values
Ethyl acetate	Chloroform/methanol (9:1)	7	1.0, 0.96, 0.90, 0.51, 0.42, 0.27, 0.14
Chloroform	Chloroform/methanol (9:1)	4	1.0, 0.96, 0.87, 0.51, 0.42
Butanol	Chloroform/methanol (9:1)	6	1.0, 0.90, 0.77, 0.51, 0.42, 0.36, 0.27, 0.18
Ethanol	Chloroform/methanol (9:1)	5	1.0, 0.96, 0.90, 0.77, 0.42, 0.27
Aqueous	Chloroform/methanol (9:1)	5	1.0, 0.96, 0.90, 0.77

R_f = Retardation factor.

mL for ethyl acetate, 14.64 μg/mL for butanol, 70.72 μg/mL for ethanol, 270.46 μg/mL for chloroform and 14.79 μg/mL for aqueous extracts. The TLC detected a total of 10 spots using chloroform/methanol (9:1) solvent (Table 4). All spots were detected using a UV detector and vanillin spray. Using chloroform/methanol (9:1) solvent most of the spots were identical, having R_f values of 1.0, 0.90, 0.77, 0.51, and 0.42. TLC-bioautography was set up using the butanol extract with *S. aureus* and *S. boydii*. With *S. aureus*, the butanol extract showed a clear zone of inhibition (78 mm long and 36 mm wide) all over the TLC plate (Figure 5). However, against *S. boydii*, the same extract showed three distinct zones of inhibition, 18 mm, 20 mm, and 12 mm, with R_f values of 0.27, 0.42, and 0.90, respectively. Synergistic effects were tested against *S. boydii* with the chloroform extract and gentamicin (FIC = 0.9). The MIC of gentamicin was found to be ≥2000 μg/mL against *S. boydii* while the MIC of the chloroform extract was 3000 μg/mL. However, on combining the chloroform with gentamicin, the MIC was recorded as 187 μg/mL (Figure 6).

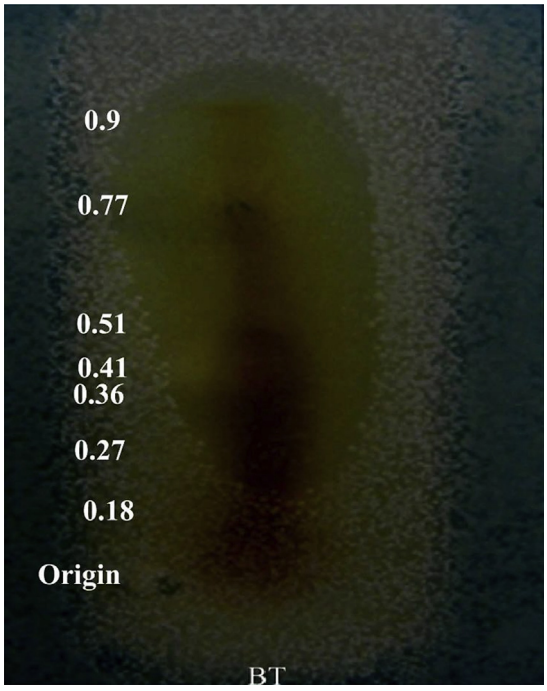

Figure 5. Thin layer chromatography-bioautography of the butanol extract against *Staphylococcus aureus*. Values represent the retardation factor (R_f). BT = butanol extract.

4. Discussion

Solvents or extraction agents used in the preparation of phyto-pharmaceuticals must be suitable for dissolving the important therapeutic drug constituents. In addition, solvents used should be easy to remove, inert, nontoxic, and nonflammable. Commonly, aqueous extracts have been used, particularly in preliminary studies. It is hypothesized that alcoholic solvents efficiently penetrate cell membranes, permitting extraction of high amount of endocellular components in contrast to solvents with lower polarity such as chloroform and petroleum ether.[15] In this way, alcohol dissolves chiefly polar constituents together with medium and low polar compounds extracted by co-solubilization. The extraction of any crude drug with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of the drug and solvent used. The determination of solvent extractive value thus permits the selection of a suitable solvent. A low yield of soluble matter can also be indicative of poor quality or contamination. The amount of material extracted per gram of powdered bulb of *E. bulbosa* ranged from 54 mg/g to 264 mg/g. Several techniques have been reported for testing of antimicrobial activity of natural products including plant extracts. However, the agar cup method is always employed for preliminary testing of

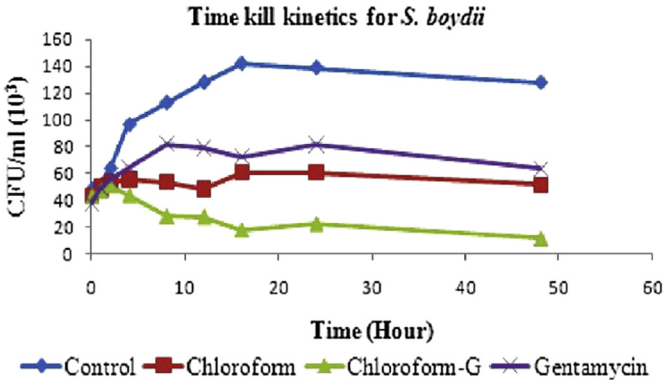

Figure 6. Time–kill kinetics of *Shigella boydii* treated with a combination of chloroform extract and gentamicin. CFU = colony forming units.

antimicrobial activities of crude extracts, whereas advanced methods like determination of the MIC are mostly used as secondary confirmation methods. In the agar cup method here, all test bacteria produced zones of inhibition with ethyl acetate, ethanol, butanol and aqueous extracts. The butanol extract was the most active followed by aqueous, ethanol, ethyl acetate and chloroform extracts. Ethyl acetate and chloroform extracts were as effective as that of ethanol and aqueous extracts but were less effective than the butanol extract.

Gram-positive bacteria were more sensitive than Gram-negative bacteria.[16] However, in most cases the commercially available antibiotic ciprofloxacin was found to exhibit higher zones of inhibition compared with the plant extracts. In addition, it was observed that the antimicrobial activity of ciprofloxacin was higher against the Gram-positive organisms compared with the Gram-negative ones. This phenomenon has been observed elsewhere and one reason for it may be the fact that Gram-negative bacteria are more resistant to the action of antimicrobials compared with their Gram-positive counterparts as a result of the more complex cell wall of the former.[17] Antimicrobial resistance has been reported for the most predominant pathogenic microorganisms like *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, and *S. typhimurium*.[18] In the present study, test organisms showed a 50% multiple antibiotic resistance (MAR) index (*P. aeruginosa*, *P. fluorescens*, *V. alginolyticus*, and *V. cholerae*), a 55% MAR index (*S. boydii*) and a 15% MAR index (*S. flexneri*). Among Gram-positive bacteria, *S. aureus* and *S. epidermidis* showed a 25% MAR index, while *B. subtilis*, *B. brevis* and *B. licheniformis* had MAR indices of 30%, 35% and 40%, respectively. All these organisms can be considered as potential pathogens to humans.

MICs are considered the gold standard for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definite answer when a borderline result is obtained by other methods or when diffusion methods are not appropriate.[19] The MIC results among Gram-positive and Gram-negative bacteria showed that ethyl acetate, butanol, ethanol, and aqueous extracts were able to prevent the growth of most of the test strains with selective activities. Within the concentration range of 46 μg/mL to 3000 μg/mL (w/v), growth of the test bacteria was inhibited. The results of the MBC assay showed that at a concentration of 3000 μg/mL (w/v), 50% of the test bacteria were killed. Also, all low MICs (< 1500 μg/mL) exhibited by extracts had MBC values four or eight times that of the MIC, in corresponding microorganisms, highlighting their interesting antimicrobial potency. From these results, it can be observed that, most of the test samples exert a killing effect on the experimental organisms.

Bioautography remains a useful technique to reveal compounds with antibacterial activity. A plant extract contains many compounds that may be seen in visible light or under UV conditions. Chromatographic spray reagents are also useful to show different types of compounds and to ascertain which types of compound are present in the plant extracts. The TLC results showed that a total of 10 spots were detected using chloroform/methanol (9:1) as the mobile phase. Among all the extracts five spots were uniformly recorded with R_f values of 1.0, 0.90, 0.77, 0.51, and 0.42. TLC-bioautography confirmed antimicrobial activity in the butanol extract against *S. aureus* and *S. boydii* through development of a clear zone of inhibition. A synergistic effect was established against *S. boydii* by combining the chloroform extract along with the standard antibiotic gentamicin. Several hypotheses can be put forward to explain the mechanism of synergy.[20] Firstly, if the bulb extract disrupts the microbial lipopolysaccharide layer, this may help in restoring porin channels, thus facilitating the flow of gentamicin to target sites. Secondly it may cause negative effects on efflux mechanisms and allow sufficient accumulation of gentamicin in the bacterium thus aiding its inhibitory activity. Thirdly, protein synthesis in the bacterium may be inhibited by administration of bulb extracts in combination with gentamicin, which may not be possible with the antibiotic or the extract alone. Fourthly, the chloroform extract of *E. bulbosa* may be blocking inhibitory effects of the enzymes or additional inhibitory effects of the plant material.

Neyestani et al[21] studied the antimicrobial effects of tea extract with certain antibiotics against *E. coli*. Different concentrations of green tea extracts were tested and results showed that green tea extract increased the antibacterial effects of gentamicin and amikacin. From their experiment they conclude that the antimicrobial effects of green tea extracts used in conjunction with certain antibiotics against *E. coli* may vary depending on the amount of the extract and the antibiotic being used. Jazani et al[22] also evaluated the synergistic effect of water-soluble green tea extract on the activity of ciprofloxacin against urinary tract isolated *E. coli*. They also confirmed that the combination of water soluble green tea extracts and ciprofloxacin had *in vitro* synergistic effects on *E. coli.* Recently, Saeidi et al[23] studied the antibacterial activity of some plant extracts against extended spectrum β-lactamase-producing *E. coli* isolates. They reported that the isolated *E. coli* showed resistance to antibiotics with an MIC of 128 mg/L for amikacin and 512 mg/L for gentamicin and ciprofloxacin. They observed the MIC values were 5 mg/mL and 2.5 mg/mL, for ethanol extracts of plants *Marrbium vulgare* and *Peganum harmala,* respectively against most of the isolated *E. coli*. All these studies are comparable and consistent with the results of the *E. bulbosa* extracts of the present experiments.

Several aromatic compounds and their glycosides such as eleutherinone, eleutherine, isoeleutherine, eleutherol, (R)-4-hydroxyeleutherin, eleuthone, isoeleuthoside C, eleutherinol 8-O-βD-glucoside are present in *E. bulbosa* bulbs.[24,25] The antibacterial activity is due to more than a few compounds as evidenced by TLC-bioautography. Consequently, further studies are required to isolate the active compounds from the butanol and alcoholic extracts of the *E. bulbosa* bulb that are responsible for the antibacterial properties, which may lead to identification of novel compounds in the field of clinical antimicrobial agents.

Conflicts of interest

The authors declare that they have no competing interests.

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